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Studies on Bulblet Differentiation in Bulb-scale Segments of *Lilium longiflorum* V. Isolation and purification of calmodulin

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Summary

Adventitious bulblet induction in lily bulb-scale segments was inhibited by a calmodulin (CaM) inhibitor, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7). This fact suggested that bulblet induction was controlled by CaM. We tried to isolate and purify CaM in lily bulbs through trichloro acetic acid precipitation, anion-exchange chromatography, and gel permiation chromatography. The content of lily CaM was 2 μg per g tissue and the molecular weight was 17,000. The amino acid sequence (the N termunus to the 25th amino acid) of lily CaM was determined. The ability of lily CaM for stimulation of rat erythrocyte membrane Ca^{2+}-ATPase activity was lower and required 3 times higher concentration of Ca^{2+} than bovine brain CaM. The CaM activity was suppressed by W-7.

Key words: bulblet differentiation, calcium, calmodulin, *Lilium longiflorum*

Introduction

In bulb-scale segments of *Lilium longiflorum,* adventitious bulblet differentiation was induced by application of naphthaleneacetic acid (NAA) and benzyladenine (BA)[8]. The bulblet differentiation was stimulated by wounding[8], traumatic acid[8], anaerobic treatment[9], phospholipid, phorbol ester[10] and calcium ionophore A23187[15]. Many physiological phenomena induced by Ca^{2+} were controlled by Ca^{2+}-binding protein, calmodulin (CaM)[4]. Adventitious bud initiation in *Torenia* stem segments was also promoted by A23187 and application of CaM inhibitor suppressed the initiation[14]. Therefore, the bulblet differntiation in lily bulb-scale segments was thought to be controlled by CaM. In higher plants, the CaMs were purified from *Hordeum vulgare*[6], *Daucus carota*[1], *Pisum sativum*[1], *Spinacea oleracea*[5], *Arachis hypogea*[2] and *Renilla reniformis*[2]. We examined the effects of CaM inhibitor on bulblet induction, and tried to isolate CaM in lily bulbs.

Materials and Methods

Effects of CaM inhibitor on bulblet induction

Plantlets of *Lilium longiflorum* Thunb. were grown in vitro as reported previously[8] and formed bulbs (about 15 mm in diameter) in the basal part of plantlets were harvested.

The outer 2 scales of these bulbs were transversally cut to 6 segments and used as explants. The explants were cultured on the basal culture medium containing Murashige and Skoog's mineral salts[13], 4 % sucrose and 0.25 % Gelrite (hereafter referred to as MS medium). Calcium ionophore A23187 (Calbiochem-Behring, USA) was dissolved in dimethylsulfoxide and added to the MS medium. The final concenration of dimethylsulfoxide was adjusted to 0.25 % in all treatments. In another series of experiments, 0.1 μM naphthaleneacetic acid (NAA) and 1 μM benzyladenine (BA) were added to the MS medium. One of CaM inhibitors, N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W-7; Seikagaku, Japan) was added to the MS medium at various concentrations with A23187, NAA, BA, traumatic acid or 12-O-tetradecanoyl phorbol-13-acetate (TPA). In the anaerobic treatment, the explants just after the excision from mother bulb-scales were exposed to 100 % N_2 stream for 1 hr, and then cultured on the MS meedium with or without W-7. The cultures were maintained under 16 hr long-day photoperiod (6,000 lux) and constant temperature of 25 ± 2 °C. After 3 weeks of culture, bulblet differentiation in the cultured explants and the number of bulblets formed in the explant were examined.

Extraction of CaM from lily bulbs

The bulbs (500 g fresh weight) were homogenized in 1,250 ml of 50 mM sodium phosphate buffer (pH 5.7) and 5 ml of 0.5 M ethylenediaminetetraacetic acid (EDTA), followed by centrifugation at 5,000 g for 30 min, and 75 ml of 50 % trichloro acetic acid (TCA) was added to the resulted supernatant. The mixture was stirred for 10 min,the pH was adjusted to 5.2 with NaOH, and 6.3 ml 0.5 M EDTA and 1.2 ml 0.1 M phenylmethylsulfonyl fluoride (PMSF) were added to the solution. The solution was stirred for 1 hr, followed by centrifugation at 5,000 g for 10 min, then added 75 ml 50 % TCA to supernatant, and further stirred for 10 min. After centrifugation at 5,000 g for 20 min, the resulted pellet was dissolved by 10 ml 1 M tris (hydroxy-methyl) aminomethane (Tris), added with 10 ml 50 mM sodium phosphate buffer and 20 μl 0.1 M PMSF, and then dialyzed against 50 mM sodium phosphate buffer with 0.2 M NaCl for 6 hr. The dialysate was centrifuged at 17,000 g for 30 min, and the resulted supernatant was referred as crude CaM fraction.

Purification of lily CaM

The crude CaM fraction was loaded onto a DEAE-cellulose column equilibrated by 50 mM sodium phosphate buffer (pH 5.7) with 0.2 M NaCl and eluted by 0.2 to 0.4 M NaCl gradient in sodium phosphate buffer. The fractions exhibiting CaM activity were collected (totally about 250 ml), 25 ml 50 % TCA was added and then stirred for 10 min. After centrifugation at 6,500 g for 10 min, the resulted pellet was dissolved by 1 M Tris, then dialyzed against 10 mM Tris-HCl (pH 8.0) for 2 hr.

The dialysate was centrifuged at 17,000 g for 20 min, and the supernatant was loaded onto a Ultrogel-AcA44 (LKB, Sweden) column equilibrated by 10 mM Tris-HCl (pH 8.0) with 0.1 M NaCl. The CaM fraction (10 ml) was collected, 1 ml 50 % TCA was added, stirred for 10 min followed by centrifugation at 7,000 g for 10 min. The pellet was dissolved

by 1 M Tris and then dialyzed against deionized water for 12 hr.

Assay for CaM activity

Calmodulin was assayed on the basis of its ability to stimulate the activity of erythrocyte membrane Ca^{2+}-ATPase. One rat was decaptated and total blood was collected. The blood was washed by 0.9 % NaCl with 1 mM ethyleneglycol-bis-(2-aminoethyl ether)-N, N, N′, N′,-tetraacetic acid (EGTA), and added with 10 vol. 20 mM imidazol -HCL buffer (pH 7.8) followed by centrifugation at 15,000 g for 30 min. The pellet was suspended to 20 mM imidazol-HCl buffer (pH 7.8) with 1 mM EGTA, and centrifuged at 15,000 g for 30 min. The resulted pellet was re-suspended to 40 mM imidazol-HCl buffer (pH 7.1) with 40 mM histidine followed by centrifugation at 15,000 g for 30 min, the pellet suspended to 4 ml 18 mM histidine-18 mM imidazol buffer (pH 7.1) was erythrocyte membrane preparation.

Assay mixture was made by equal volume of 18 mM histidine-18 mM imidazol buffer (pH 7.1), 0.1 mM EGTA, 80 mM NaCl, 3 mM $MgCl_2$, 15 mM KCl, 0.2 mM $CaCl_2$ and 0.1 mM ouabain (Aldrich, USA). The 0.25 ml assay mixture, 0.1 ml 3 mM ATP, 0.05 ml erythrocyte membrane preparation and 0.1 ml sample were mixed, pre-incubated at 30 °C for 30 min, and 3.9 ml 6 % $HClO_4$, 0.4 ml amydol reagent (1 % amydol with 20 % $NaHSO_3$) and 8.3 % ammonium molybdate were added. After incubation for 20 min, absorbance of 660 nm was measured.

Others

Native-polyacrylamide gel electrophoresis (PAGE) was performed using 15 % acrylamide without denaturing of protein. The protein sample was pre-incubated with 0.1 mM $CaCl_2$ or 0.1 mM EGTA solution. The amount of protein was estimated by Coomassie Brilliant Blue G-250 binding assay[3]. Analytical sodium dodecyl sulfate (SDS) -PAGE was conducted according to the procedure of Laemmli[12] on 15 % polyacrylamide gels in the presence of 15 mM EGTA. The purified lily CaM and standard bovine brain CaM (Amano, Japan) were prepared for SDS-PAGE by heating at 95 °C for 5 min in the presence of 1 % SDS, 1 % mercaptoethanol, and 15 mM EGTA. Cytochrome c monomer (12,400), dimer (24,800), trimer (37,200), tetramer (49,600), and hexamer (73,800) were employed as molecular weight marker. Amino acids sequence of N terminus in the purified CaM was determined with an automatic sequenator

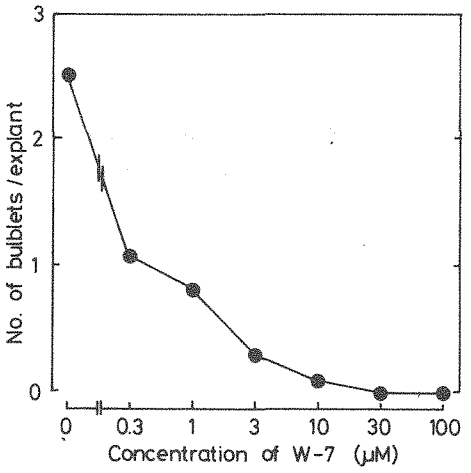

Fig. 1 Effects of W-7 on bulblet differentiation in lily bulb-scale segments.
The lily bulb-scale segments were cultured on the MS medium with 0.1 μM NAA and 1 μM BA and contained various concentrations of W-7.

Table. 1 Effects of W-7 on bulblet differentiation induced by some chemicals and anaerobic treatment in lily bulb-scale segments.

Chemicals or Treatment	No. of bulblets/explant		
	−W−7	+W−7 (1 μM)	+W−7 (10 μM)
NAA (0.1 μM) +BA (1 μM)	2.3	0.8	0.1
A23187 (1 μM)	6.4	1.6	0.4
Traumatic acid (1 μM)	4.6	1.2	0.2
TPA (0.1 μ)	9.8	2.2	0.4
Anaerobic treatment (1 hr)	5.6	2.0	0.2

The lily bulb-scale segments were cultured on the MS medium containing NAA, BA, A23187, traumatic acid or TPA with or without W-7. In another series of experiments, the segments were exposed to N_2 stream for 1 hr and then cultured on the MS medium with or without W-7.

Table. 2 Calmodulin content in lily and animal tissues.

Materials	Calmodulin content	
	μg/g tissues	μg/mg protein
Lily bulb	2	0.37
Bovine brain	406	15.9
Rat skeletal muscle	28	0.87

The lily CaM content was estimated by activation of rat erythrocyte membrane Ca^{2+}-ATPase by crude extract. The CaM contents of bovine brain and rat skeletal muscle were due to previous report[7].

(Applied Biosystems, USA; 477A).

Results and Discussion

Effects of W-7 on bulblet induction

Adventitious bulblet differentiation in the explants cultured on the medium containing NAA and BA was strongly suppressed by application of W-7 (Fig. 1). The 50 % inhibition was apparent in 0.1 μM W-7, and addition of 10 μM W-7 completely inhibited bulblet induction. Similar inhibitory effects of W-7 were shown in some differentiation-stimulating treatments, such as A23187, traumatic acid, TPA, or anaerobic treatment (Table 1). We have previously demonstrated that all of above treatments increased intracellular Ca^{2+} concentration and bulblet differentiation in lily bulb-scale segments could be regulated by intracellular Ca^{2+} level[10, 15]. In animal and microbial cells, intracellular Ca^{2+} was acted to some physiological phenomena through activation of a Ca^{2+}-binding protein, CaM[4]. The fact that CaM inhibitor, W-7, suppressed bulblet differentiation (Fig. 1, Table 1) suggested that CaM is a main regulator for bulblet induction.

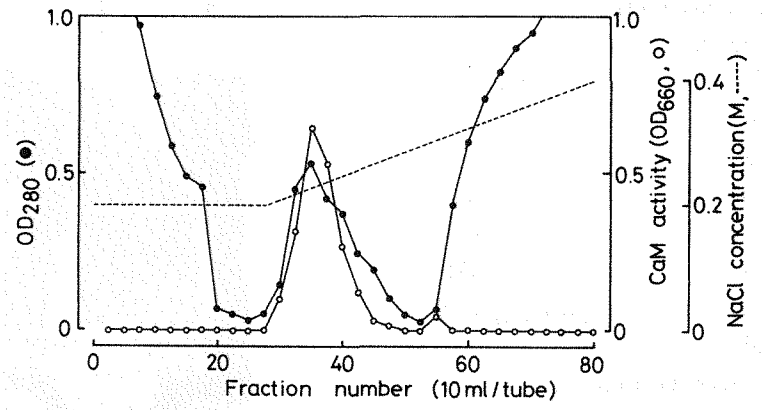

Fig. 2 Anion exchange chromatography profile of lily proteins. The crude extract from lily bulbs were fractionated by TCA and the 6% TCA precipitates were loaded onto a DEAE-cellulose chromatography. The concentration of NaCl (·····) in elute was changed. The CaM activity (○) was assayed by stimulation of Ca^{2+}-ATPase activity.

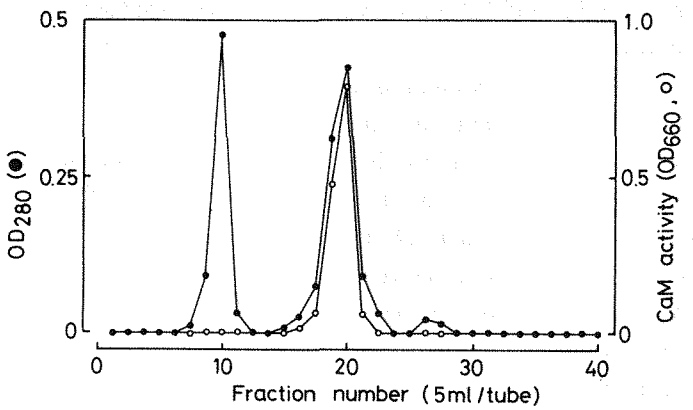

Fig. 3 Gel permiation chromatography profile of lily proteins. The fractions possesed CaM activity were loaded onto a Ultrogel-AcA44 chromatography. The CaM activity (○) was assayed by stimulation of Ca^{2+}-ATPase activity.

Table. 3 Purification of lily CaM.

Fraction	Protein content (mg)	CaM content (mg)	CaM content (μg/mg protein)	Purification (times)
Crude extract*	26,390	9.8	0.37	1.0
3% TCA sup	11,860	9.6	0.81	2.2
6% TCA ppt	900	9.2	10.2	27.6
DEAE-cellulose chromatography	50	8.4	161	434
Ultrogel-AcA44 chromatography	9	7.3	776	2092

*Crude extract was prepared from 5 kg of lily bulbs.

Purification of lily CaM

Five kg fresh weight of lily bulbs were used for CaM extraction. The 26.4 g soluble proteins were extracted and the content was about 5.28 mg per g fresh weight. The crude extract contained 9.8 mg CaM (0.37 μg CaM per mg protein) and the content was lower than animal CaM (Table 2); the CaM contents were 15.9 μg in bovine brain and 0.07 μg per mg protein in rat skeletal muscle[7]. The crude extract was fractionated with 3 % and 6 % TCA; the lily CaM could be fractionated in the supernatants of 3 % TCA and in the precipitates of 6 % TCA. The supernatants of 3 % TCA contained 11.9 g proteins and 9.6 mg CaM, and the precipitates of 6 % TCA contained 900 mg proteins and 9.2 mg CaM. After TCA fractionation, the CaM fraction was applied to ion-exchange chromatography using DEAE-cellulose column and the fraction showing CaM activity (fraction No. 34 to 52) were collected (Fig. 2). The fraction contained 50 mg proteins and 8.4 mg CaM. The fraction was applied to gel permiation chromatography using Ultrogel -AcA44 column ant the sharp single peak was appeared in the elution profile (Fig. 3). The peak was collected and the protein and/or CaM contents were measured. Finally, about 9 mg proteins and 7.3 mg CaM were harvested. The total purification step of lily CaM was summarized in Table 3, and the result from native-PAGE was shown in Fig. 4.

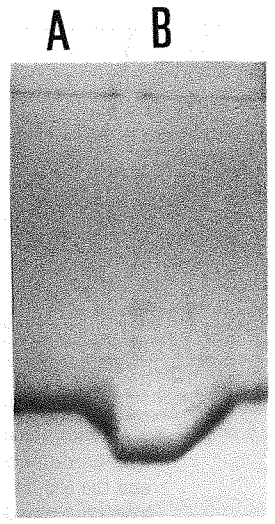

Fig. 4 Native-PAGE profiles of purified lily CaM.
The purified lily CaM was pre-incubated with 0.1 mM $CaCl_2$ (A) or 0.1 mM EGTA (B) solution for 2 hr, and then applied to native-PAGE.

Table. 4 The molecular weight of CaMs in some plant and animal tissues.

Materials	Molecular weight (daltons)
Lily bulbs	17,000
Barley leaves[6]	16,700
Spinach leaves[5]	18,000
Bovine brain[16]	17,500

The molecular weight of lily CaM was estimated from SDS-PAGE.

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          1                   5                  10
Lily   : Ala-Gln-Gln-Leu-Thr-Asp-Glu-Gln-Ile-Ala-Glu-Phe-Lys-
Bovine: Ala-Asp-Gln-Leu-Thr-Glu-Glu-Gln-Ile-Ala-Glu-Phe-Lys-

              15                  20                  25
Lily   : Glu-Ala-Phe-Ser-Leu-Phe-Asp-Lys-Asp-Gly-Asp-Gly-
Bovine: Glu-Ala-Phe-Ser-Leu-Phe-Asp-Lys-Asp-Gly-Asp-Gly-
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Fig. 5 Amino acid sequence on N terminus in lily CaM.
Amino acid sequece on N terminus in lily CaM was determined and compared with the sequence in bovine brain CaM[11].

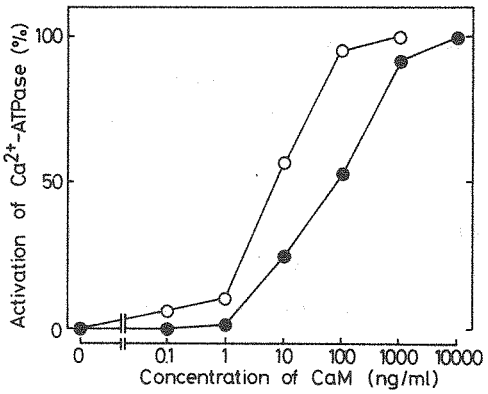

Fig. 6 Activation of Ca^{2+}−ATPase by lily or bovine CaM.
Various concentrations of lily (●) or bovine (○) CaM were added to the assay mixture and Ca^{2+}-ATPase activation by CaM were measured. The activities of lily and bovine CaMs were expressed as relative activity compared with the control assay mixture contained 1 μg/ml bovine brain CaM and 14 μM Ca^{2+}.

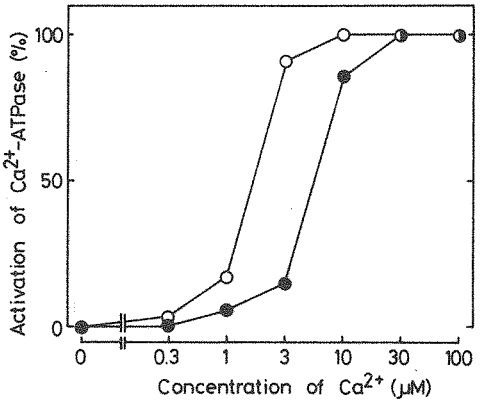

Fig. 7 Requirement of Ca^{2+} for Ca^{2+}-ATPase activation by CaM.
Various concentrations of $CaCl_2$ were added to the assay mixture, and Ca^{2+}-ATPase activation by lily (●) or bovine (○) CaM were measured. The activities of lily and bovine CaMs were expressed as relative activity compared with the control assay mixture contained 1 μg/ml bovine brain CaM and 14 μM Ca^{2+}.

Characteristics of lily CaM

The molecular weight of lily CaM estimated from SDS-PAGE was 17,000 daltons. The weight was higher than barley CaM (16,700)[6] and lower than bovine (17,500)[16] and spinach CaM (18,000)[5] (Table 4).

Amino acid sequence of the N terminus in lily CaM was determined. As shown in Fig. 5, amino acid sequence in lily CaM was almost similar to bovine brain CaM[11], the 2nd and 6th amino acids were only changed from asparagine and glutamic acid to glutamine and asparagine.

The lily CaM was assayed on the basis of its ability to stimulate the activity of rat erythrocyte membrane Ca^{2+}-ATPase. The stimulating ability of lily CaM was 10 times lower than bovine brain CaM (Fig. 6). The presence of Ca^{2+} was required for the activation of Ca^{2+}-ATPase by CaM. In above experiments, the assay mixture contained 14 μM Ca^{2+}. Thus, the effects of Ca^{2+} concen-

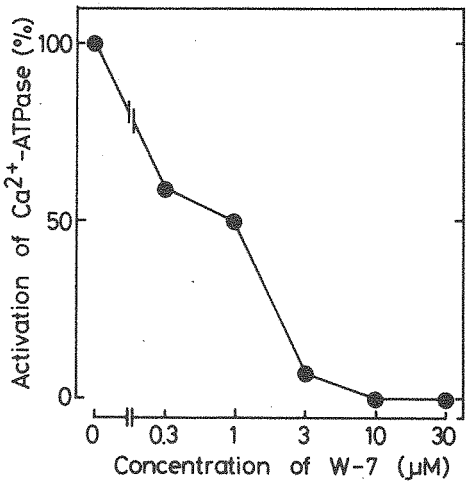

Fig. 8 Effects of W-7 on lily CaM activity.
Various concentrations of W-7 were added to the assay mixture, and Ca^{2+}-ATPase activation by lily CaM were measured. The activity of lily CaM was expressed as relative activity compared with the control assay mixture contained 1 μg/ml bovine brain CaM and 14 μM Ca^{2+}.

tration in the assay mixture on activity of lily and bovine brain CaM were examined. As shown in Fig. 7, the lily CaM required 3 times higher concentration of Ca^{2+} than bovine brain CaM. These facts seemed to suggest that animal (rat) Ca^{2+}-ATPase was more easily stimulated by animal (bovine) CaM, and the animal (bovine) CaM required lower concentration of Ca^{2+} for activation than plant (lily) CaM.

The effects of W-7 on the lily CaM activity were also examined. The addition of W-7 into assay mixture remarkably suppressed CaM activity (Fig. 8). The inhibition pattern was closely related to the suppression of bulblet induction by W-7 (Fig. 1).

Efforts are directed to obtain more information regarding isolation of genes encoding lily CaM, and mechanism of action of Ca^{2+}-CaM during adventitious bulblet induction in lily bulb-scale segments.

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鉄砲ユリの鱗片切片培養における球根分化に関する研究
V. カルモデュリンの単離・精製

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摘　　要

鉄砲ユリの鱗片切片から球根分化は細胞内Ca^{2+}濃度の上昇によって引き起こされるが，Ca^{2+}結合蛋白質であるカルモデュリン(CaM)に対する阻害剤のW-7を添加することで抑制される。このことはCaMが球根分化を制御していることを示唆している。そこで鉄砲ユリの球根からのCaMの単離・精製を試みた。その結果，トリクロロ酢酸による分画，陰イオン交換クロマトグラフィー，ゲル浸透クロマトグラフィー等を経てCaMを精製することができた。CaM含量はg新鮮重あたり約2 μgであり，分子量は17,000であった。このCaMのN末端から25番目までのアミノ酸配列を決定した。ラット赤血球膜のCa^{2+}-ATPaseの活性化を指標としてCaM活性を測定したが，鉄砲ユリのCaMは牛脳のCaMに比較して活性は1/10であり，活性化には牛のCaMよりも3倍の濃度のCa^{2+}を必要とした。このCaM活性はW-7により完全に阻害された。